Cancer Research

Long Noncoding RNA LINC00092 Acts in Cancer-Associated Fibroblasts to Drive Glycolysis and Progression of Ovarian Cancer

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Abstract

The majority of patients with epithelial ovarian cancer are diagnosed at a late stage when the peritoneal metastases exist; however, there is little knowledge of the metastatic process in this disease setting. In this study, we report the identification of the long noncoding RNA LINC00092 as a nodal driver of metastatic progression mediated by cancer-associated fibroblasts (CAF). Prometastatic properties of CAFs *in vitro* and *in vivo* were found to associate with elevated expression of the chemokine CXCL14. In clinical specimens, elevated levels of CXCL14 in CAFs also correlated with poor prognosis. Notably, CXCL14-high CAFs mediated upregulation of LINC00092 in ovarian cancer cells, the levels of which also correlated with poor prognosis in patients. Mechanistic studies showed that LINC00092 bound a glycolytic enzyme, the fructose-2,6-biphosphatase PFKFB2, thereby promoting metastasis by altering glycolysis and sustaining the local supportive function of CAFs. Overall, our study uncovered a positive feedback loop in the metabolism of CXCL14-positive CAFs and ovarian cancer cells that is critical for metastatic progression. *Cancer Res; 77(6); 1369–82. ©2017 AACR.*

Introduction

Epithelial ovarian cancer constitutes a major gynecologic malignancy, with a reported incidence rate of 3–12/100,000 women annually (1, 2). As early symptoms of ovarian cancer are often clinically atypical or absent, the majority of ovarian cancer patients are diagnosed at a late stage. This condition underscores the urgency of early detection of these patients and establishment of new therapeutic avenues for successful intervention. Considering that the predominant biological characteristic that differentiates malignant from benign tumors is the ability to metastasize, it is necessary to identify novel metastasis-related molecules for ovarian cancer.

Recently, the interactions between cancer cells and components of tumor microenvironment have been highlighted in cancer metastasis (3). Cancer-associated fibroblasts (CAF) are one of the most abundant cell components in tumor entities and emerging evidence has highlighted the role of CAFs in promoting carcinogenesis and cancer progression in different cancer cell types, including ovarian cancer (3–5). However, it is not yet clear which factors define the different subtypes of CAFs with heterogeneous molecular identities and functions. CXCL14 was recently found to be a key factor for the cancer-promoting properties of CAFs (6–8). However, the role of CXCL14-secreting CAFs and their downstream molecular effectors in cancer cells in ovarian cancer pathogenesis and progression remain to be unraveled.

Long noncoding RNAs (lncRNA) belong to a class of noncoding RNA over 200 nucleotides with no protein-coding capacity. They are actively involved in different physiologic and pathologic processes including development, immune response, and tumorigenesis (9, 10). In recent years, lncRNA expression is frequently reported to be dysregulated in different cancer types and is correlated with cancer aggressiveness (11). In particular, the lncRNAs that were found to be functionally important for ovarian cancer include NEAT1 (12), UCA1 (13), ZNF300P1 (14), and AB073614 (15). However, few studies have been conducted to explore whether and how CAFs modulate ovarian cancer progression through lncRNAs. LncRNAs control pleiotropic biological processes through interactions with other cellular molecules including DNA, protein, and RNA. In particular, previous studies have proven that lncRNAs could regulate glycolysis in cancer cells,

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-16-1615

a defining hallmark of cancer (16), either by directly binding with key glycolytic enzymes (17) or as a result of enhanced transcription of glycolytic enzyme genes activated by lncRNA's binding with RNA polymerase II (18). These observations lead us to postulate whether CAFs promote ovarian cancer progression through lncRNA-mediated enhanced glycolysis.

In this study, we demonstrated that CXCL14 was a prometastatic factor released by CAFs in ovarian cancer. Through lncRNA microarray analysis, we found a number of lncRNAs dysregulated in ovarian cancer upon CXCL14 stimulation. Among the upregulated lncRNAs, we characterized the functional role of lncRNA LINC00092 in ovarian cancer metastasis. LINC00092 was correlated with aggressiveness of ovarian cancer *in vivo* and *in vitro* and clinical outcome of ovarian cancer patients, interacted with 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2), and involved in increased glycolysis levels in ovarian cancer metastasis. This glycolytic phenotype of ovarian cancer cells, in turn, sustained the CAFs-like features of fibroblasts within tumor microenvironment.

Materials and Methods

Patients and specimens

Fifty-eight samples of *de novo* serous ovarian cancer were recruited in this study. In addition, 25 normal ovarian surface tissue specimens as controls were collected from women who underwent oophorectomy for nonmalignant conditions in West China Second Hospital, Sichuan University (Chengdu, P.R. China). All of these samples were obtained by experienced gynecologists and examined by experienced pathologists who confirmed the diagnosis of disease samples. This study was approved by the Institutional Ethics Committee of Sichuan University. Informed consents were obtained from all patients prior to analysis.

Cell culture

SKOV-3 was obtained from ATCC and maintained in RPMI1640 (Gibco) containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin in a humid atmosphere with 5% CO_2 at 37°C. A2780 cells was obtained from European Collection of Cell Cultures and cultured in RPMI1640 containing 10% FBS and 100 U/mL penicillin–streptomycin mixture (both from Gibco-BRL) at 37°C and 5% CO_2. Cell lines received in 2010 were tested for authenticity in 2012 (SKOV-3 and A2780s) using short tandem repeat (STR) genotyping. CAFs and NAFs were isolated from same ovarian site in 10 EOC and 10 noncancerous prophylactic oophorectomy specimens, respectively, as described previously (19). Detailed procedure for isolation of fibroblasts could be seen in Supplementary Methods.

Plasmid and RNAi transfection

Detailed descriptions of plasmids, shRNAs and siRNAs, and cell transfection procedures can be found in the Supplementary Methods.

Ovarian cancer survival analysis in datasets

Kaplan–Meier survival analyses for disease outcomes in Australian Ovarian Cancer Study (AOCS) dataset ($n = 285$), The Cancer Genome Atlas (TCGA) dataset ($n = 565$), and Mateescu's cohort ($n = 107$) were conducted using the online database (www.kmplot.com). The clinical stages of the patients in these three cohorts range from FIGO stage I to stage IV. *P* values were calculated with log-rank (Mantel–Cox) test. Patients were stratified into "low" and "high" expression based on autoselect best cutoff in the database.

LncRNA microarray

The Human LncRNA Microarray V3.0 (Arraystar Inc.) is designed for the global profiling of human lncRNAs and protein-coding transcripts. The microarray work was performed by KangChen Bio-Tech. The arrays were scanned by the Agilent Scanner G2505B (Agilent Technologies) and the acquired array images were analyzed by Agilent Feature Extraction software (version 10.7.3.1; Agilent Technologies). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). The microarray results were uploaded into Gene Expression Omnibus (GEO) database as GSE82059.

RNA pull-down assay

The biotin-labeled LINC00092 and the antisense RNA were *in vitro* transcribed with a Biotin RNA Labeling Mix (Roche) and the T7 RNA polymerase (Roche), treated with RNase-free DNase I (Roche), and purified with an RNeasy Mini Kit (Qiagen). Detailed descriptions of RNA pull down procedure can be found in the Supplementary Methods.

NMR measurement of metabolites

For the measurement of metabolites in media studies, we collected conditioned media from cells treated with control and si-LINC00092 for 48 hours. Cell pellets were used for the measurement of intracellular metabolite levels. Analyses of samples were done using nuclear magnetic resonance (NMR) spectroscopy.

Peritoneal metastasis ovarian cancer nude mice model

Animal studies were reviewed and approved by the Institutional Ethics Committee of Sichuan University. The female athymic BALB/c nude mice (6–8 weeks old, 18–20 g each) were applied to establish the intraperitoneal xenograft tumor model of human ovarian cancer as described previously (20). The number of metastatic nodules was counted and ascites volumes were measured at sacrifice.

F2,6BP and lactate measurements

A total of 1×10^4 cells were trypsinized and washed twice with PBS prior to the measurement of total intracellular F2,6BP. The F2,6BP concentration was normalized to total cellular protein as measured by the bicinchoninic acid (BCA) assay. Lactate levels were measured using a lactate oxidase–based colorimetric assay read at 540 nm according to the manufacturer's instructions (Beyotime) and normalized to cell numbers.

Antibody array

CAFs (1×10^5) and A2780s cells (1×10^5) were cultured alone or together in 2 mL of RMPI1640 complete medium in a 6-well plate. Twenty-four hours later, cells were washed twice with PBS and incubated with fresh serum-free RPMI1640 for another 24 hours. Then, the coculture conditioned media (CM) of CAFs together with either control A2780s cells or PFKFB2-silenced A2780s cells were collected for antibody array. The human cytokine/chemokine array kits (Ray Biotech Inc.) were

used to detect a panel of 24 secreted cytokines and chemokines according to the manufacturer's recommended protocol.

Statistical analysis

The data are presented as the means ± SD of three independent experiments unless otherwise indicated. GraphPad Prism (GraphPad Software Inc.) was applied for data analysis with all data assessed for normal distribution and equal variance. The correlation analysis was analyzed using a linear regression analysis. Comparisons between two groups were performed Student *t* test, and differences among multiple groups were evaluated by one-way ANOVA. The survival of different treatment groups were analyzed by Kaplan–Meier analysis. Differences were considered statistically significant at $P < 0.05$.

Results

CXCL14 is overexpressed in CAFs of metastatic lesion of ovarian cancer and predicts clinical outcome

We first investigated whether CAFs exerted impact on ovarian cancer metastasis compared with NAFs. Our results showed that CAFs promoted the metastatic capacity of both A2780s ovarian cancer cells (Supplementary Fig. S1A) and SKOV-3 ovarian cancer cells (Supplementary Fig. S1B) compared with NAFs. Mice inoculated with A2780s mixed with CAFs demonstrated poorer survival compared with those inoculated with A2780s mixed with NAFs ($P = 0.024$, Supplementary Fig. S1C). Similar results were also observed in the SKOV-3 model ($P = 0.011$, Supplementary Fig. S1D). At sacrifice, we found more metastatic nodules in the group of mice inoculated with A2780s mixed with CAFs compared with A2780s mixed with NAFs ($P < 0.001$, Supplementary Fig. S1E). The same results were also seen in the SKOV-3 model ($P < 0.001$, Supplementary Fig. S1F). These results were consistent with previous findings (19, 21, 22) and implicated that CAFs could promote peritoneal metastasis of ovarian cancer.

To explore the underlying mechanisms for CAF-mediated ovarian cancer metastasis, we examined a panel of chemokines and cytokines previously reported to be important for CAFs in both CAFs and NAFs. Interestingly, we found that CXCL14 was the most significantly upregulated chemokine in CAFs derived from ovarian cancer compared with NAFs (Fig. 1A). We next extended validation of CXCL14 expression in CAFs in a larger cohort of ovarian cancer and normal ovary. Interestingly, we found that CXCL14 mRNA levels were significantly increased in CAFs of ovarian cancer with metastasis compared with NAFs and CAFs without metastasis ($P < 0.0001$, Fig. 1B). Further immunoblotting analysis revealed CXCL14 expression in CAFs of ovarian cancer with metastasis was remarkably higher than that in CAFs of ovarian cancer without metastasis (Fig. 1C). As CXCL14 is a chemokine secreted to tumor microenvironment, we wondered whether CXCL14 level was also elevated in CAF-CM. Data showed that CXCL14 levels in CAF-CM in patients with metastasis were significantly increased compared with those in the CAF-CM without metastasis or those in NAF-CM ($P < 0.0001$, Fig. 1D). Immunohistochemical analysis of CXCL14 expression in ovarian cancer and normal ovary demonstrated that while no obvious positive staining was observed in normal ovary and glandular cells of ovarian cancer specimens either with or without metastasis, we noted positive immunostaining in the stroma of ovarian cancer tissues with metastasis while no immunostaining was observed in the stroma of ovarian cancer without metastasis ($P < 0.0001$, Fig. 1E). To further examine the clinical importance of CXCL14 in ovarian cancer patients, we performed Kaplan–Meier analysis in ovarian cancer patients of AOCS and TCGA. Our results showed that CXCL14 expression was negatively correlated with overall survival of ovarian cancer patients in AOCS dataset [$P = 0.0018$, HR = 2.07 (1.3–3.3), Fig. 1F] and TCGA dataset [$P = 0.0037$, HR = 1.57 (1.2–2.1), Fig. 1G]. However, as these studies did not separately assess CXCL14 expression in tumor and tumor stroma, it was still unclear whether this overexpressed CXCL14 derived from tumor cells or tumor stroma. Thus, we attempted to explore other public ovarian cancer datasets to determine this.

Tan and colleagues recently constructed a transcriptomic microarray database of 3,431 human ovarian cancers named "CSIOVDB," which included clinicopathologic parameters and follow-up information of ovarian cancer patients (23). We observed in CSIOVDB database that while no significantly differences of CXCL14 expression were observed between normal ovary surface epithelium (OSE) and ovarian tumor, there was significant differences of CXCL14 expression between ovarian tumor and peritoneal metastatic sites ($P < 0.01$, Supplementary Fig. S2A). In addition, CXCL14 expression was significantly upregulated in tumor stroma compared with normal ovary stroma ($P < 0.001$, Supplementary Fig. S2A). Moreover, CSIOVDB analysis revealed that CXCL14 was significantly overexpressed in ovarian cancers with more advanced FIGO stage ($P < 0.05$, Supplementary Fig. S2B), higher differentiation degree ($P < 0.01$, Supplementary Fig. S2C), and refractory or resistant disease ($P < 0.05$, Supplementary Fig. S2D). Consistent with results from AOCS and TCGA datasets, Kaplan–Meier analysis of ovarian cancer patients in CSIOVDB also showed that CXCL14 expression was negatively correlated with overall survival of ovarian cancer patients ($P = 0.0035$, Supplementary Fig. S2E). In addition, CXCL14 expression was correlated with epithelial-to-mesenchymal transition (EMT) scores ($P = 5.18 \times 10^{-39}$, Supplementary Fig. S2F) and a mesenchymal subtype of ovarian cancer ($P < 0.001$, Supplementary Fig. S2G). Collectively, these data showed that CXCL14 is overexpressed in CAFs of ovarian cancer with metastasis and predicts clinical outcome.

CAF-secreted CXCL14 maintains metastatic phenotype of ovarian cancer

We next examined the biological functions of CAF-secreted CXCL14 in ovarian cancer. Compared with A2780s cells in medium alone and cocultured for 8 hours with NAFs, the number of invading cancer cells upon coculturing for 8 hours with CAFs significantly increased (Fig. 2A and B). To determine whether CAF-secreted CXCL14 contributes to ovarian cancer cell invasion, we employed a polyclonal anti-human CXCL14 antibody and two CXCL14-siRNAs to inhibit CXCL14 function in CAFs. In the presence of CAFs, the number of invasive cancer cells was significantly reduced by adding anti-human CXCL14 antibody in a dose-dependent manner, but not by an isotype-matched IgG at 10 mg/mL (Fig. 2A). However, anti-human CXCL14 antibody itself did not exert any impact on the invasion of ovarian cancer cells in culture medium alone or cocultured with NAFs that do not express CXCL14 (Fig. 2A). Transfection of CAFs with either of the two CXCL14-siRNAs significantly reduced the number of invasive cancer cells (Fig. 2B). In contrast, treatment of A2780s cells with recombinant CXCL14 (rCXCL14) for 8 hours enhanced the invasion of ovarian cancer cells in a dose-dependent manner

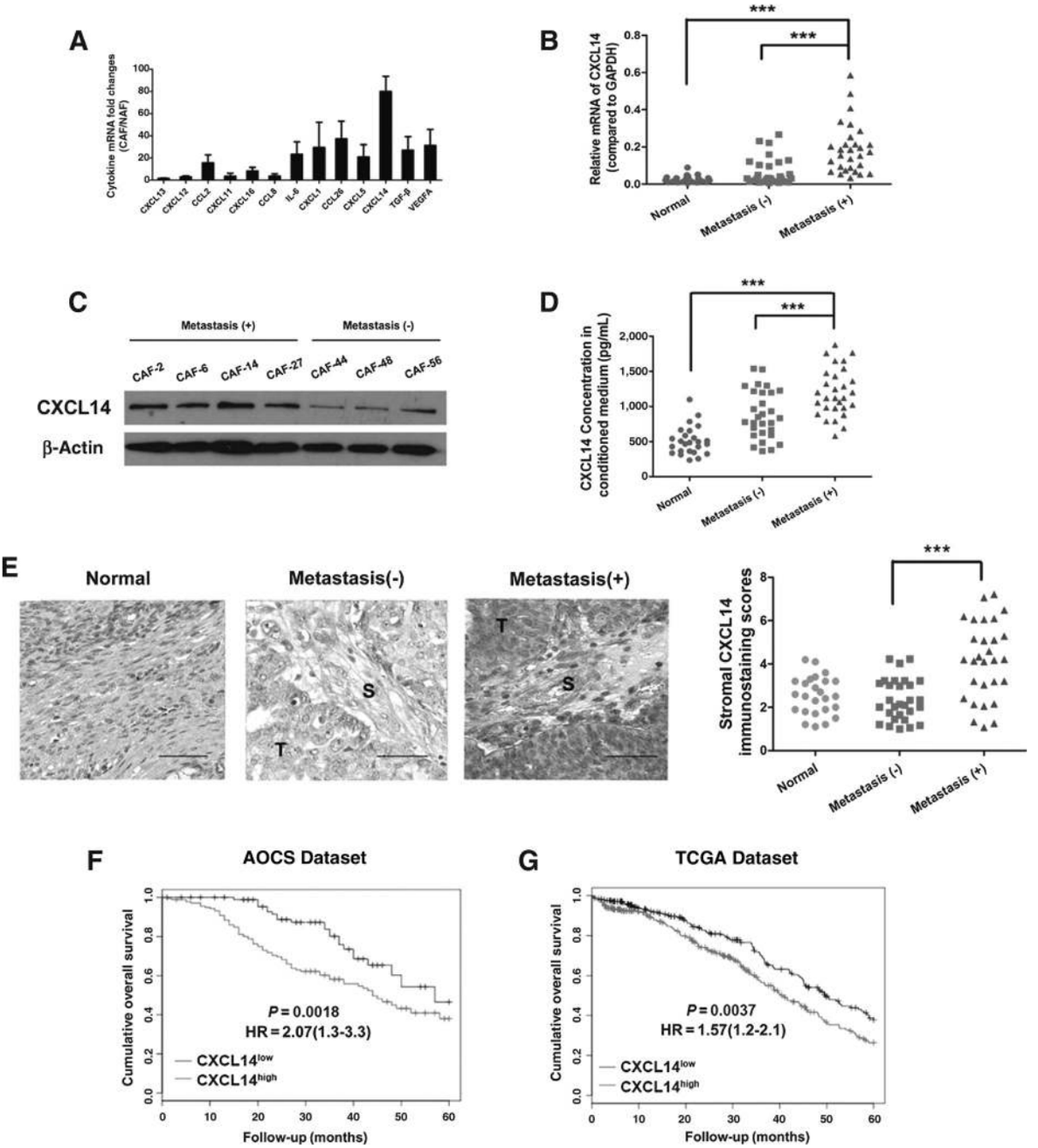

Figure 1.

Overexpression of CXCL14 in CAFs of metastatic lesion of ovarian cancer. **A,** mRNA levels of selected cytokines between CAFs and NAFs in ovarian cancer. **B,** mRNA levels of CXCL14 in normal ovary, ovarian cancer without metastasis, and ovarian cancer with metastasis. **C,** CXCL14 protein levels in CAFs of ovarian cancer with and without metastasis. **D,** CXCL14 levels in the NAF-CM of normal ovary, CAFs of ovarian cancer without metastasis, and CAFs of ovarian cancer with metastasis. **E,** Immunohistochemical analysis of CXCL14 in specimens of normal ovary, ovarian cancer without metastasis, and ovarian cancer with metastasis. **F,** Kaplan–Meier analysis of AOCS patients with ovarian carcinoma showing a significant correlation between CXCL14 protein expression and overall survival ($n = 285$). **G,** Kaplan–Meier analysis of TCGA patients with ovarian carcinoma showing a significant correlation between CXCL14 protein expression and overall survival ($n = 565$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scale bar, 50 μm.

(Fig. 2C). Moreover, ovarian cancer cells incubated with CM from CAFs transfected with the two CXCL14-siRNAs also demonstrated significantly decreased migratory capacity (Fig. 3A) and increased anoikis rate ($P < 0.05$, Fig. 3B).

In vivo data showed that mice inoculated with A2780s cells mixed with CXCL14 KD CAFs demonstrated significantly decreased peritoneal metastasis compared with those inoculated with A2780s cells mixed with control CAFs (Fig. 3C), in the aspect

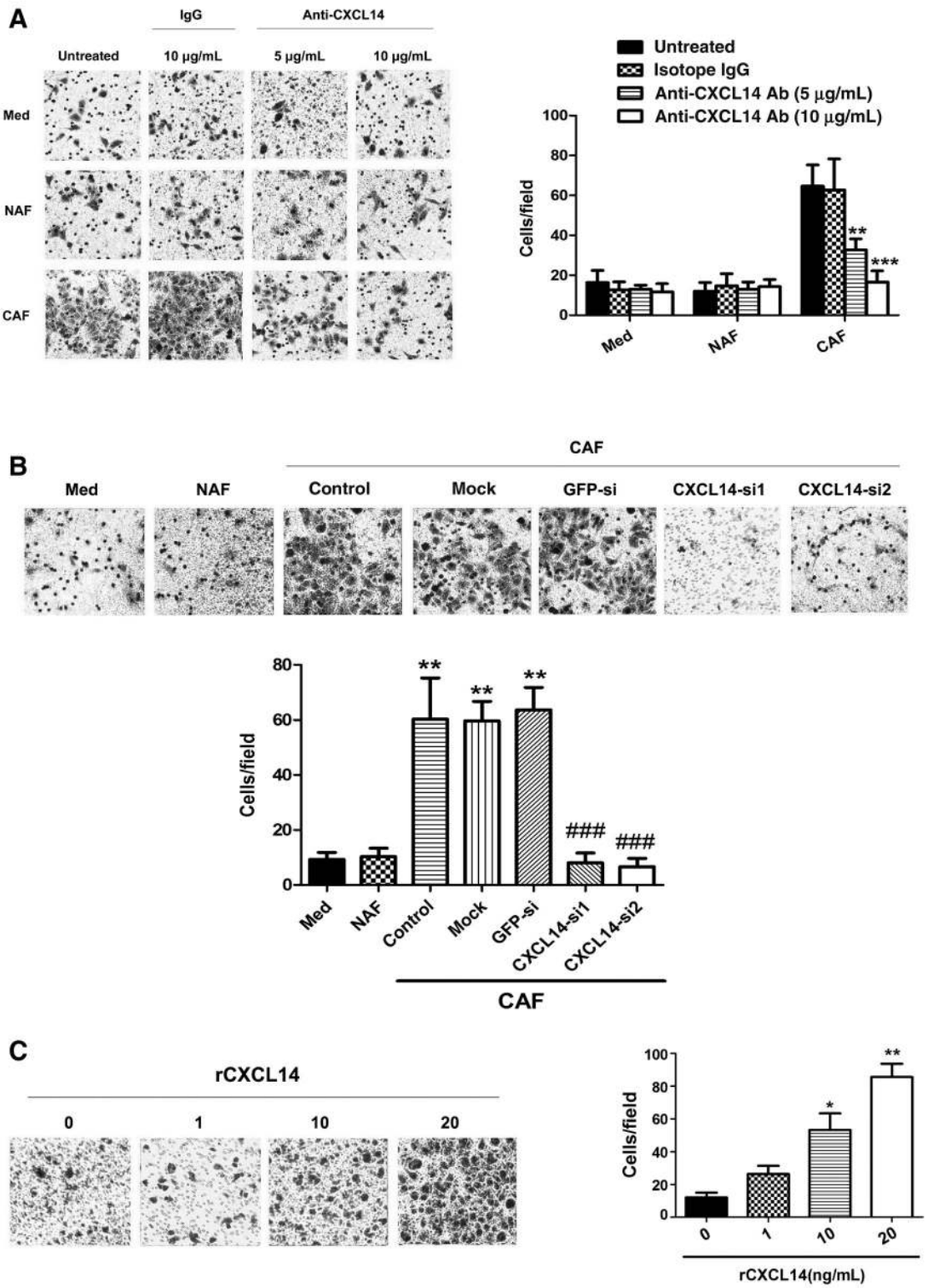

Figure 2.

CAFs in ovarian cancer promote the invasion of ovarian cancer cells via CXCL14. **A,** Transwell assay for A2780s cells plated on the top cell culture inserts, with control culture medium alone (Med), NAF, or CAF plated in the bottom chambers in the presence or absence of an anti-CXCL14 antibody at 5 or 10 mg/mL, or an isotype-matched IgG control (IgG). **B,** Similar to **A**, A2780s cells were cocultured with control culture medium alone (Med), NAF, or CAF that were treated with control (control), mock transfected, or transfected with either of the two CXCL14-siRNAs or GFP-siRNA. **C,** Transwell assay for A2780s cells with rCXCL14 at increasing concentrations (1–20 ng/mL) added to culture medium in the bottom chambers. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

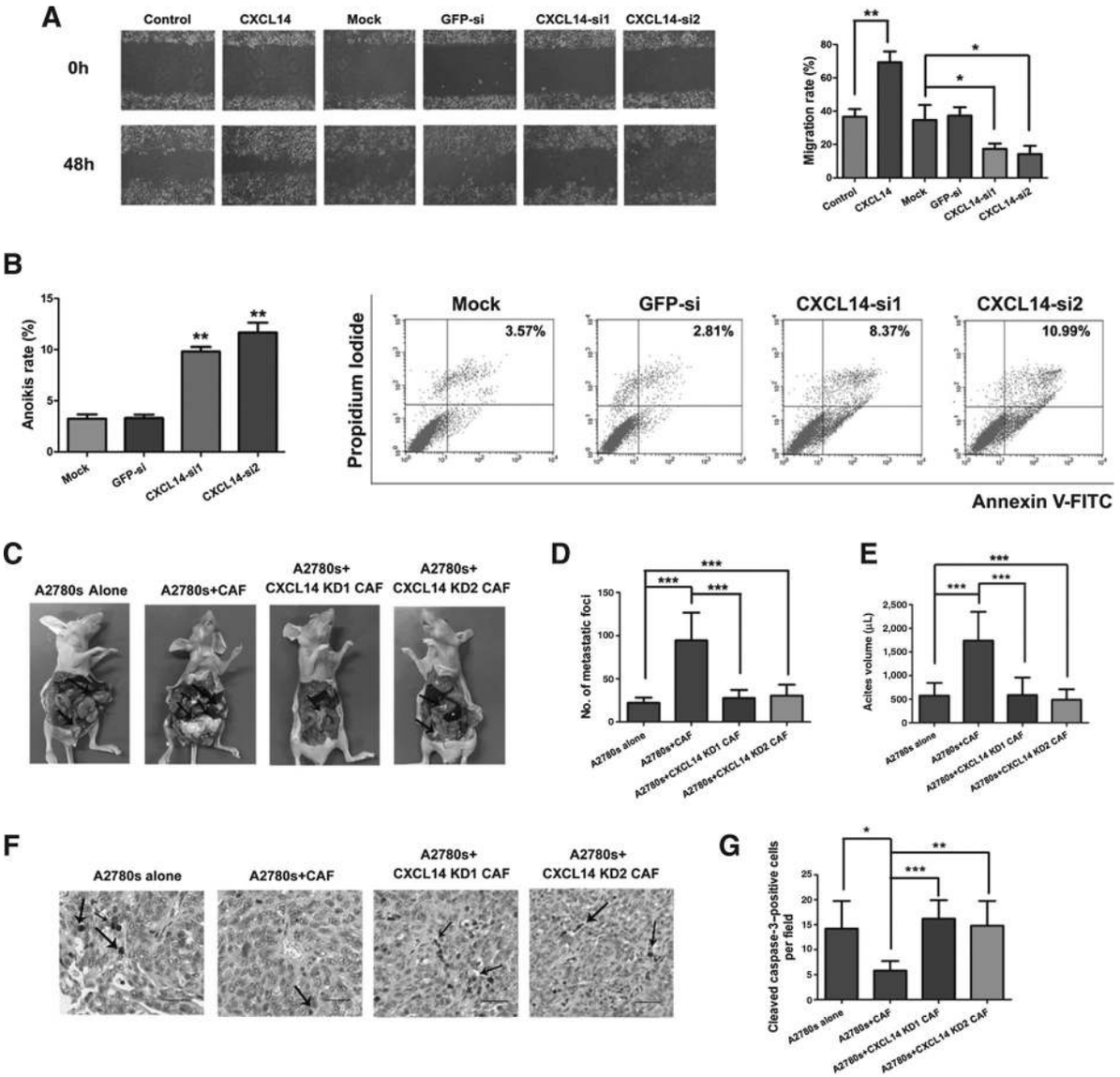

Figure 3.

CXCL14 promotes ovarian cancer metastasis *in vitro* and *in vivo*. **A,** Wound healing analysis of A2780s cells cocultured with control culture medium alone (control), recombinant CXCL14 protein, or CM from CAFs that were mock transfected (mock) or transfected with either of the two CXCL14-siRNAs or GFP-siRNA. **B,** Relative anoikis rate of A2780s cells cocultured with CM from CAFs that were mock transfected (mock) or transfected with either of the two CXCL14-siRNAs or GFP-siRNA. Representative flow cytometry plot for each group was shown. **C,** Representative pictures of peritoneal metastasis in an orthotopic model generated by intrabursal injection of A2780s ovarian cancer cells alone or together with either CAFs or CXCL14-silenced CAFs ($n = 10$ in each group). **D,** Box plot of number of metastastic nodules of tumors in the abdominal cavities in the group of A2780s cells alone, A2780s cells together with CAF group, and A2780s cells together with CXCL14-silenced CAF group ($n = 10$ in each group). **E,** Box plot of the ascites volumes collected from the abdominal cavities in the group of A2780s cells alone, A2780s cells together with CAF group, and A2780s cells together with CXCL14-silenced CAF group ($n = 10$ in each group). **F,** Representative IHC photographs of cleaved caspase-3 in the tumor specimens obtained from peritoneal metastasis foci. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scale bar, 50 μm. **G,** Box plot of number of cleaved caspase-3-positive cells per field in the group of A2780s cells alone, A2780s cells together with CAF group, and A2780s cells together with CXCL14-silenced CAF group ($n = 10$ in each group).

of number of metastatic nodules ($P < 0.001$, Fig. 3D) and ascites volume ($P < 0.001$, Fig. 3E). Immunohistochemical analysis of cleaved caspase-3 in the tissues collected with *in vivo* models demonstrated that the level of cleaved caspase-3 was significantly increased in the CXCL14 KD group compared with that in the control group (Fig. 3F). In addition, we further used anti-human CXCL14 antibody to investigate the potential clinical applicability of CXCL14 inhibition in the treatment of ovarian cancer. We compared the cancer metastasis of mice inoculated with A2780s control cells combined with control CAFs either systemically administered with anti-human CXCL14 antibody or IgG. Data showed that systemic administration of anti-CXCL14 antibody

significantly decreased ovarian cancer metastasis compared with control (Supplementary Fig. S3A), in terms of prolonged survival time ($P = 0.029$, Supplementary Fig. S3B), decreased ascites volume ($P < 0.01$, Supplementary Fig. S3C), and reduced the number of metastatic nodules ($P < 0.01$, Supplementary Fig. S3D). Therefore, these observations suggest that CAFs promote the invasiveness of ovarian cancer cells via CXCL14.

LINC00092 is induced upon stimulation by CAF-secreted CXCL14 in ovarian cancer

As CAFs demonstrated potent prometastatic properties in ovarian cancer, we aimed to characterized the downstream molecular events responsible for CAF-mediated ovarian cancer metastasis. LncRNAs are proved functionally important noncoding RNAs that recently emerge as key players in ovarian cancer development and progression. However, no existing evidence characterized the relevance of dysregulation of lncRNAs in ovarian cancer cells upon interaction with CAFs. Thus, we further examined whether CAF-secreted CXCL14 could induce downstream alterations of lncRNAs in ovarian cancer using lncRNA microarray (Fig. 4A). Among the differential lncRNAs identified, we selected five top upregulated lncRNAs (RP11-119F7.5, ZEB1-AS1, XLOC_000493, RP11-12L2.4, and LINC00092) upon treatment with recombinant CXCL14 protein in A2780s ovarian cancer cell line for functional characterization by siRNA-mediated knockdown analysis. We found that only LINC00092 knockdown significantly decreased the migration ability of A2780s ovarian cancer cell line (data not shown) and thus we chose LINC00092 as a downstream target of CAF-secreted CXCL14 for further exploration. LINC00092 was significantly increased in A2780s cells incubated either with CAF-CM ($P < 0.01$, Fig. 4B) or with recombinant CXCL14 protein ($P < 0.01$, Fig. 4C), compared with those either with NAF-CM or control, respectively. Moreover, significantly increased mRNA level of LINC00092 was observed in tissues of ovarian cancer with metastasis compared with tissues of ovarian cancer without metastasis and normal ovary ($P < 0.001$, Fig. 4D). Further analysis of human ovarian cancer specimens demonstrated that LINC00092 expression in cancer epithelial cells were correlated with immunostaining scores of stromal CXCL14 in corresponding specimens ($P = 0.013$, $r^2 = 0.1728$, Fig. 4E), as revealed by representative immunostaining graphs of stromal CXCL14 expression in LINC00092-high and LINC00092-low ovarian cancer tissues (Fig. 4F). Kaplan–Meier analysis in Mateescu's ovarian cancer cohort (24) demonstrated that LINC00092 expression level was correlated with overall survival ($P = 0.0041$, Fig. 4G) and progression-free survival ($P = 0.038$, Fig. 4G) of ovarian cancer patients. These data suggest that LINC00092 might be the downstream effector of CAF-secreted CXCL14 that might be involved in ovarian cancer metastasis.

LINC00092 promotes ovarian cancer progression both *in vitro* and *in vivo* and is involved in altered level of glycolysis

We next characterized the functional role of LINC00092 in ovarian cancer metastasis. We designed two siRNAs targeting LINC00092 and transfected A2780s cell line with these two siRNAs (Fig. 5A). Our data showed that LINC00092-silenced ovarian cancer cells demonstrated significantly reduced invasive capacity ($P < 0.01$, Fig. 5B) and increased anoikis rate ($P < 0.001$, Fig. 5C). *In vivo* experiments showed that LINC00092-silenced ovarian cancer cells showed significantly compromised metastatic potential compared with control (Fig. 5F), revealed by decreased ascites volume ($P < 0.001$, Fig. 5D) and reduced the number of metastatic nodules ($P < 0.001$, Fig. 5E). Moreover, the overall survival of mice inoculated with LINC00092-silenced A2780s cells was significantly longer than that of mice inoculated with control A2780s ($P < 0.05$, Fig. 5G). Immunohistochemical analysis of cleaved caspase-3 in the tissues collected with *in vivo* models demonstrated that the level of cleaved caspase-3 was significantly increased in the LINC00092-silenced group compared with that in the control group (Supplementary Fig. S4). To validate whether LINC00092 overexpression could could enhance ovarian cancer migration regardless of CAF-secreted CXCL14 stimulation, we compared the difference of migratory abilities between LINC00092-overexpressing A2780s cells and control A2780s cells, both of which were incubated with CXCL14-silenced CAF-CM. We found that LINC00092-overexpressing A2780s cells demonstrated significantly increased migratory capacity compared with control A2780s cells, even without stimulation of CAF-secreted CXCL14 ($P < 0.001$, Supplementary Fig. S5). This finding indicated that the effects of CXCL14 knockdown in CAFs could be rescued by LINC00092 overexpression in ovarian cancer cells.

LINC00092 binds with PFKFB2 in ovarian cancer metastasis

As recently, Warburg effects have been identified to play an important role in tumorigenesis and cancer metastasis (25, 26) and lncRNAs have been proven to regulate Warburg effects via different mechanisms (17, 18), we further examined whether LINC00092 was linked to altered glycolysis in ovarian cancer. We found that after knockdown of LINC00092, the metabolites in cancer cells (intracellular) and CM showed a significant reduction in several metabolites compared with controls (Fig. 5H and I). Among the metabolites changed, lactate showed the greatest decrease (Fig. 5H and I). These results demonstrated that LINC00092 might alter the level of glycolysis in ovarian cancer metastasis.

We next explored the possible mechanism responsible for the role of LINC00092 in altering glycolysis in ovarian cancer metastasis. As recent studies have reported that lncRNAs exert molecular functions through binding to specific molecules, we explored whether LINC00092 participated in ovarian cancer metastasis through interacting with specific proteins. We first examined the relative mRNA levels of key glycolytic enzymes in ovarian cancer cells that overexpress LINC00092 compared with control. Data demonstrated that only the mRNA levels of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2), hexokinase-1 (HK-1), and lactate dehydrogenase A (LDHA) were significantly upregulated in ovarian cancer cells that overexpress LINC00092 compared with control (Fig. 6A). Then, three independent RNA pull-down assays were performed to further verify the association between LINC00092 and the three glycolytic enzymes. Interestingly, only PFKFB2 was detected by Western blotting (Fig. 6B). The EZH2 protein, which has been reported to physically associate with lncRNAs (MALAT1 as positive control to interact with EZH2; ref. 27), and has been validated to have no association with LINC00092 by RNA immunoprecipitation (RIP) analysis (Supplementary Fig. S6), was included as a negative control in the Western blotting assay (Fig. 6B). Furthermore, we analyzed the relationship between mRNA level of LINC00092 and PFKFB2 immunostaining scores in ovarian cancer patients and found a significant positive correlation between mRNA level of LINC00092 and PFKFB2 immunostaining scores ($r^2 = 0.2662$,

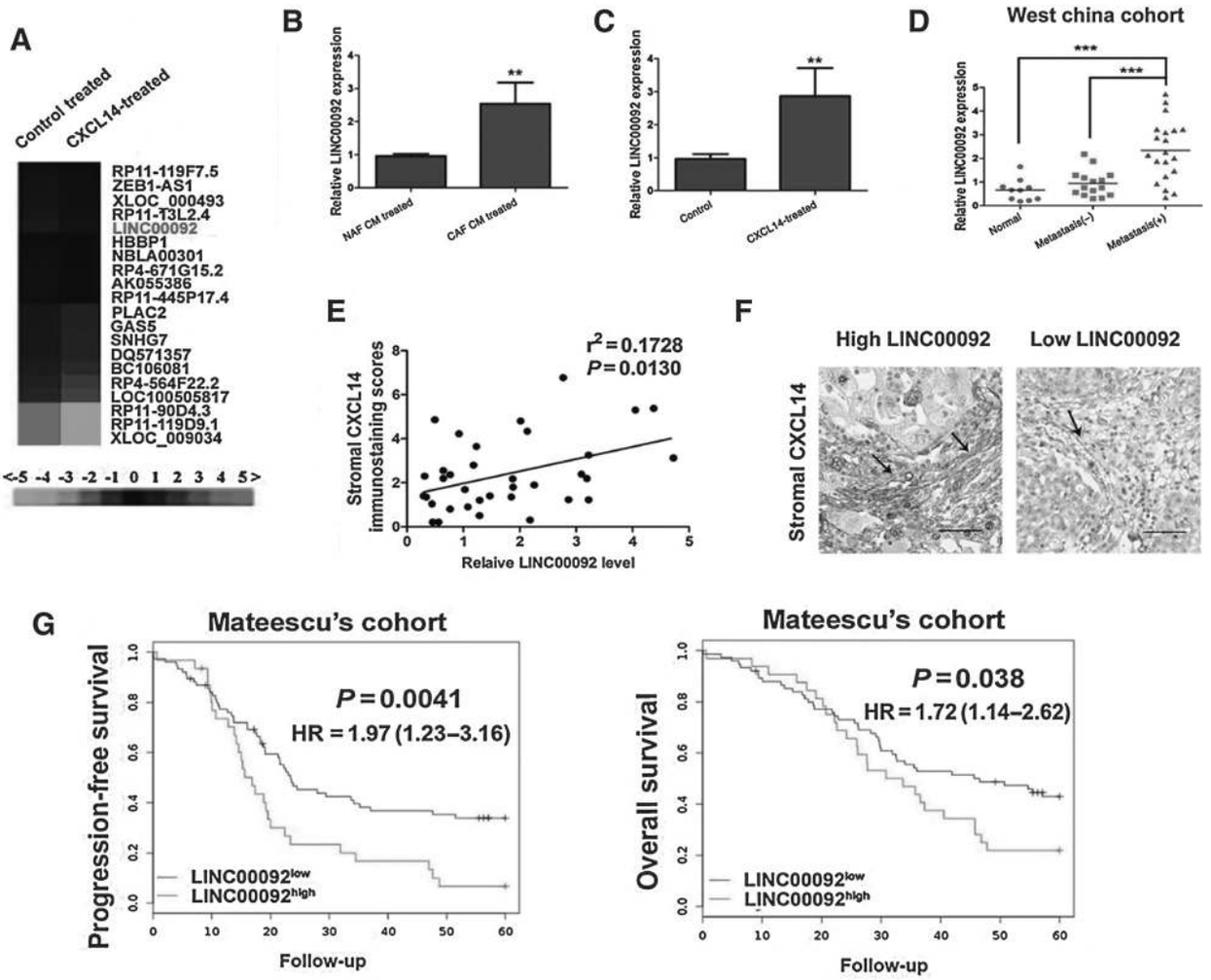

Figure 4.

CXCL14-positive CAFs induce overexpression of LINC00092 in ovarian cancer. **A,** Cluster maps of top upregulated or downregulated lncRNAs in A2780s ovarian cancer cell line treated with recombinant CXCL14 protein or control. Expression of lncRNAs upregulated in the CXCL14-treated group and the downregulated proteins are shown. **B,** Relative levels of LINC00092 in A2780s ovarian cancer cell line cultured in CAF-CM and NAF-CM, respectively. **C,** Relative levels of LINC00092 in A2780s ovarian cancer cell line cultured in CAF-CM and NAF-CM, respectively. **D,** Relative LINC00092 levels in specimens from normal ovary, ovarian cancer patients with metastasis, and ovarian cancer patients without metastasis. **E,** Correlation between relative LINC00092 level and stromal CXCL14 immunostaining scores in ovarian cancer patients with linear regression lines and Pearson correlation significance. **F,** Representative immunostaining graphs of stromal CXCL14 expression in LINC00092-high and LINC00092-low ovarian cancer specimens. We have stratified the ovarian cancer clinical samples into two categories, including LINC00092-high group and LINC00092-low group, based on median LINC00092 expression value as the cutoff. **G,** Kaplan–Meier analysis of ovarian cancer patients showing a significant correlation between LINC00092 expression and progression-free survival and overall survival in Mateescu's cohort ($n = 107$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scale bar, 50 μm.

$P = 0.0015$, Supplementary Fig. S7A). The levels of PFKFB2 mRNA were also significantly reduced in LINC00092 knocked down ovarian cancer cells (Supplementary Fig. S7B). These observations indicated that LINC00092 binds with PFKFB2 in ovarian cancer upon metastasis.

The role of PFKFB2 in the maintenance of metastatic and glycolytic phenotype of ovarian cancer

We further analyzed the correlation of expression of the three glycolytic enzymes with overall survival of ovarian cancer patients in TCGA datasets and found that among the three, only PFKFB2 was significantly negatively correlated with overall survival of ovarian cancer patients [$P = 0.036$, HR = 1.36 (1.02–1.83), Fig. 6C]. Considering the results from RNA pull-down and survival analysis, we focused on the functional role of PFKFB2 in ovarian cancer metastasis. We found that PFKFB2 expression was significantly higher in tissues of ovarian cancer with metastasis compared with normal ovary tissues and tissues of ovarian cancer without metastasis (Fig. 6D). In CSIOVDB dataset (Fig. 6E) and TCGA dataset (Fig. 6F), we also observed significantly upregulated PFKFB2 expression in ovarian cancer. Moreover, PFKFB2 expression was confirmed to be upregulated in tissues of advanced stage

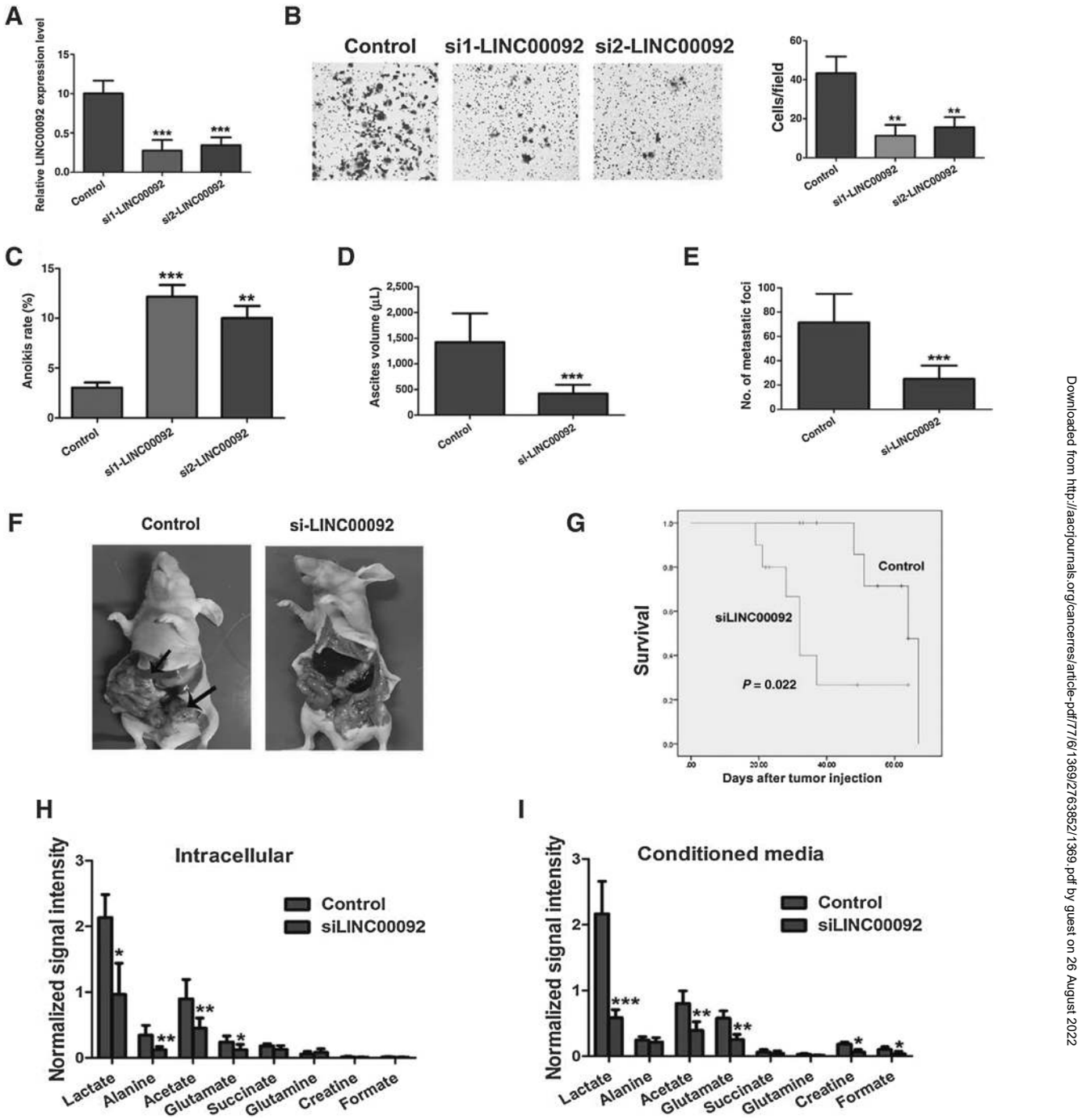

Figure 5.

LINC00092 promotes ovarian cancer progression and is involved in the altered level of glycolysis. **A,** Relative LINC00092 levels in control A2780s cell line and A2780s cell lines transfected with two LINC00092 siRNAs. **B,** Transwell assay for A2780s cells transfected with control and two LINC00092 siRNAs, respectively. **C,** Relative anoikis rate in A2780s cells transfected with control and two LINC00092 siRNAs, respectively. **D,** Box plot of the ascites volumes collected from the abdominal cavities of an orthotopic model generated by intrabursal injection of control A2780s cells and LINC00092-silenced A2780s cells, respectively. **E,** Box plot of number of metastatic nodules in the abdominal cavities of an orthotopic model generated by intrabursal injection of control A2780s cells and LINC00092-silenced A2780s cells, respectively. **F,** Representative pictures of peritoneal metastasis in mice inoculated with control A2780s cells and LINC00092-silenced A2780s cells, respectively. **G,** Kaplan–Meier analysis of mice inoculated with control A2780s cells and LINC00092-silenced A2780s cells, respectively ($n = 20$ in each group). **H** and **I,** Metabolites levels in control and siLINC00092-treated cells. Data from intracellular and CM measured via NMR method are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

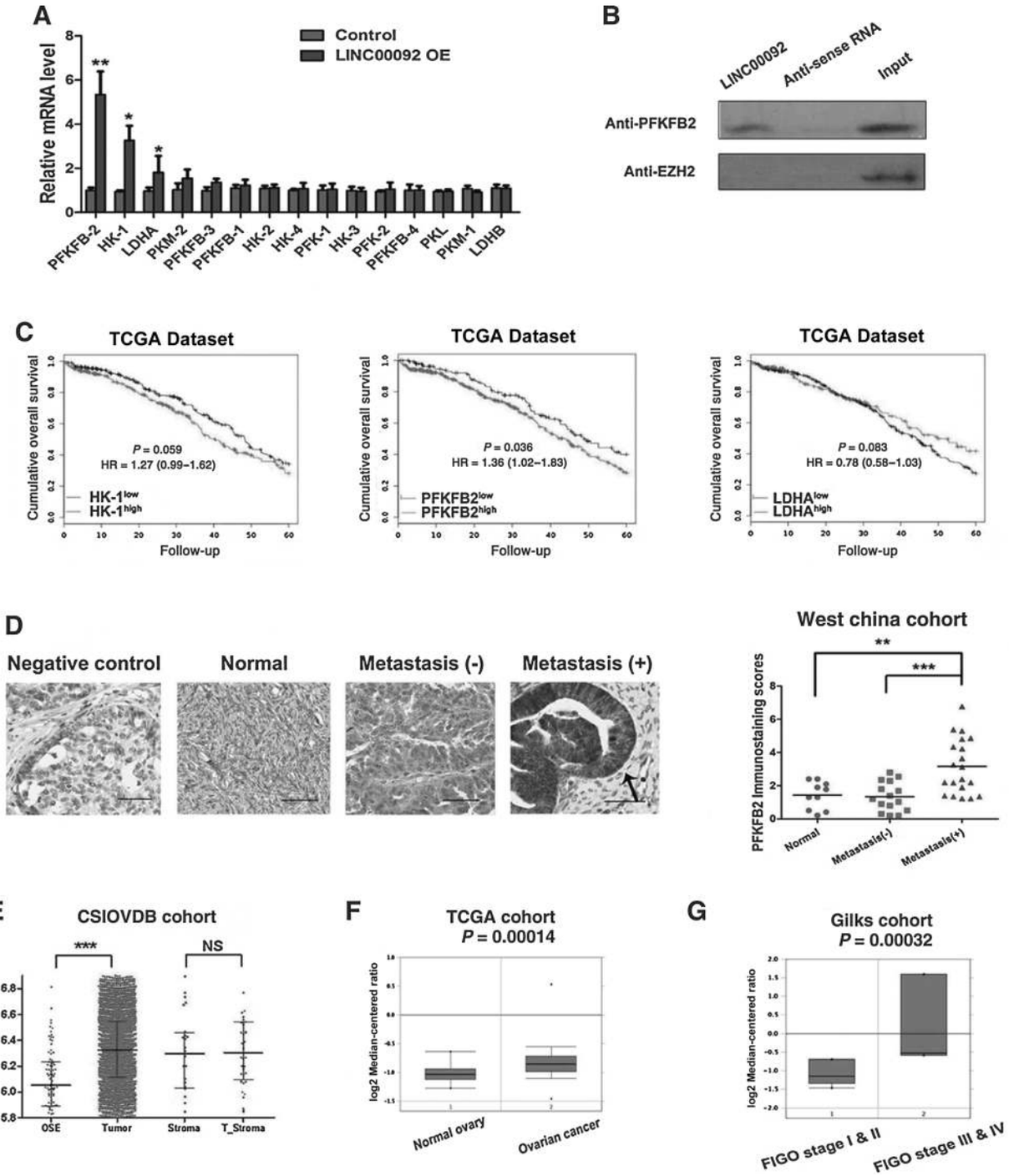

Figure 6.

LINC00092 binds with PFKFB2 to promote ovarian cancer metastasis. **A,** Relative mRNA levels of key glycolytic enzymes in control A2780s cell line and LINC00092-overexpressing (OE) A2780s cell line. **B,** Western blotting analysis of RNA pull-down assays from A2780s cellular extracts was performed. An Ab against the EZH2 protein was used as the negative control. **C,** Kaplan–Meier analysis of TCGA patients with ovarian carcinoma for the correlation between HK-1, PFKFB2, and LDHA protein expression and overall survival ($n = 565$). **D,** Immunohistochemical analysis of PFKFB2 in specimens of normal ovary, ovarian cancer without metastasis, and ovarian cancer with metastasis (normal group $n = 10$, ovarian cancer without metastasis group $n = 15$, ovarian cancer with metastasis group $n = 20$). **E,** The expression of PFKFB2 in ovarian surface epithelium (OSE), ovarian tumor, normal ovary, stroma and ovarian tumor stroma in CSIOVDB analysis. **F,** The expression of PFKFB2 in normal ovary and ovarian cancer in TCGA dataset. **G,** The expression of PFKFB2 in early-stage (FIGO stage I and II) ovarian cancer and advanced stage (FIGO stage IIII and IV) in Gilks cohort. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scale bar, 50 μm.

ovarian cancer (FIGO stage III and IV) compared with those of early-stage ovarian cancer (FIGO stage I and II) in Gilks cohort (Fig. 6G). These results implicated that PFKFB2 might be critical for ovarian cancer metastasis.

We next assessed the functions of PFKFB2 in ovarian cancer metastasis *in vitro* and *in vivo*. PFKFB2-silenced ovarian cancer cells demonstrated significantly increased anoikis rate ($P < 0.05$, Supplementary Fig. S8A) and invasive capacity ($P < 0.05$, Supplementary Fig. S8B). Wound healing analysis demonstrated that the migratory ability of ovarian cancer cells was dramatically impaired after PFKFB2 knockdown (Supplementary Fig. S8C). In an *in vivo* ovarian cancer metastasis model, we observed that PFKFB2-silenced ovarian cancer cells showed significantly compromised metastatic potential (Supplementary Fig. S8D) revealed by both the number of metastatic nodules ($P < 0.01$, Supplementary Fig. S8E) and the ascites volume ($P < 0.01$, Supplementary Fig. S8F). Moreover, we further evaluated whether the impact of LINC00092 knockdown on the migratory capacity of ovarian cancer could be rescued by PFKFB2 overexpression. We found that PFKFB2 overexpression could efficiently offset the inhibitory effects of LINC00092 knockdown on A2780s cells, as revealed by both Transwell (Supplementary Fig. S9A) and wound healing analyses (Supplementary Fig. S9B). These data validated the functional importance of PFKFB2 in the metastasis of ovarian cancer.

We further conducted *in vivo* experiments that explore the sufficiency of each factor in the establishment of ovarian cancer metastasis using ovarian cancer cells with or without PFKFB2 or LINC00092 in combination with CAFs with or without CXCL14. We found that inoculation of ovarian cancer cells with silenced expression of either LINC00092 or PFKFB2 combined with CAFs either with or without CXCL14 could significantly inhibit ovarian cancer metastasis (Supplementary Fig. S10A), in terms of ascites volume (Supplementary Fig. S10B) and number of metastasis foci (Supplementary Fig. S10C). This phenomenon indicated that each of the three factors is indispensable for the establishment of ovarian cancer metastasis.

Glycolytic phenotype of ovarian cancer cells reciprocally sustains the hallmark of CAFs

As CAFs exert significant impact on the metastatic hallmark of ovarian cancer, we wondered whether the Warburg effects in ovarian cancer could influence the phenotype of fibroblasts. Because the product of PFKFB2, fructose-2,6-bisphosphate (F2, 6BP), could activate another critical enzyme 6-phosphofructo-1-kinase (PFK-1) and facilitate glycolysis process, we examined whether PFKFB2 could influence glycolysis in ovarian cancer. We first evaluated the knockdown efficiency of two siRNAs targeting PFKFB2 using Western blotting and found that si1-PFKFB2 was more efficient than si2-PFKFB2 (Fig. 7A) and thus we used si1-PFKFB2 in further functional studies. We observed that LINC00092 knockdown markedly inhibited lactate production (Fig. 7B) and F2,6BP production (Fig. 7C), similar to the results in the PFKFB2 knockdown group. Next, we cultured CAFs with CM from either control ovarian cancer cells or PFKFB2-silenced ovarian cancer cells and analyzed the α-SMA expression levels of CAFs. We found that the α-SMA expression was significantly decreased in CAFs cultured with CM from PFKFB2-silenced ovarian cancer cells compared with control (Fig. 7D). The mRNA levels of ACTA2, FAP, IL6, CXCL12, and VEGFA, the defining markers of CAFs, in CAFs cultured with CM from PFKFB2-silenced ovarian cancer cells were significantly reduced compared with control (Fig. 7E). Moreover, antibody array analysis revealed that the relative concentrations of IL6, TIMP-1 and VEGF, which are important factors for maintenance of cancer hallmarks, were significantly reduced in the coculture-CM of CAFs and PFKFB2-silenced ovarian cancer cells compared with coculture-CM of CAFs and control ovarian cancer cells (Fig. 7F). These data suggest that the glycolytic phenotype of ovarian cancer cells reciprocally sustains CAF-like features and contributes to the malignant characteristics of tumor microenvironment (Supplementary Fig. S11).

Discussion

Tumor microenvironment, consisting of tumor cells and tumor stroma, has recently been proven to contain an autocrine–paracrine communication circuit that reinforces cancer metastasis via reciprocal signaling (3). As one of the most abundant cells in tumor stroma, CAFs provide a supportive microenvironment for and induce aggressive behaviors of cancer cells (28, 29). In this study, we confirmed that CAFs from ovarian cancer could promote cancer metastasis. The observation that CAF-CM could prominently enhance motility and invasion of ovarian cancer cells indicated that this prometastatic effect of CAFs could be in a paracrine manner. However, the underlying molecular mechanisms remained to be defined.

CAFs around the cancer regions usually exert their tumor-supporting functions by the secretion of cytokines and inflammatory mediators. For instance, CAFs could secrete urokinase-type plasminogen activator to activate matrix-degrading protease that can cleave pro-MMPs to upregulate MMP activity, resulting in enhanced angiogenesis and metastasis. Apart from extracellular matrix remodeling, CAFs also secrete cytokines, such as FSP1 and hepatocyte growth factor, to promote tumor metastasis, which are not found in NAFs (30, 31). In our study, we found that CAFs express and secrete higher level of CXCL14 than NAFs. CXCL14, also termed BRAK, MIP-2γ, or BMAC, is an orphan member of the CXC chemokine subfamily. Recently, CXCL14 was found to be a novel CAF-derived factor favorable for cancer development and progression (7, 8). Our study demonstrated that CAFs-secreted CXCL14 promoted ovarian cancer metastasis both *in vivo* and *in vitro* and was correlated with clinical outcome. Systemic administration of anti-human CXCL14 antibody significantly inhibited ovarian cancer metastasis, raising the potential of clinical application for CXCL14 inhibition in the treatment ovarian cancer patients.

In addition to coding genes, lncRNAs open a new avenue for understanding of biological processes, which can be regulated by a series of cytokines and chemokines (32). As CAF-secreted CXCL14 act on ovarian cancer cells in a paracrine manner and downstream effectors in ovarian cancer cells remain undetermined, we further examined whether CAF-secreted CXCL14 could cause lncRNA expression alterations in ovarian cancer cells to promote cancer progression. Our data identified LINC00092 as a key upregulated lncRNA in ovarian cancer cells when treated with CXCL14 from CAFs. LINC00092 is located on the intergenic regions of 9q22.32 with no functional annotations previously. We showed that LINC00092 was overexpressed in metastatic lesions of ovarian cancer, associated with poor prognosis and correlated with stromal CXCL14 expression in ovarian cancer specimens. Functionally, LINC00092 was not only essential for ovarian cancer cell invasion and migration *in vitro*, but also was

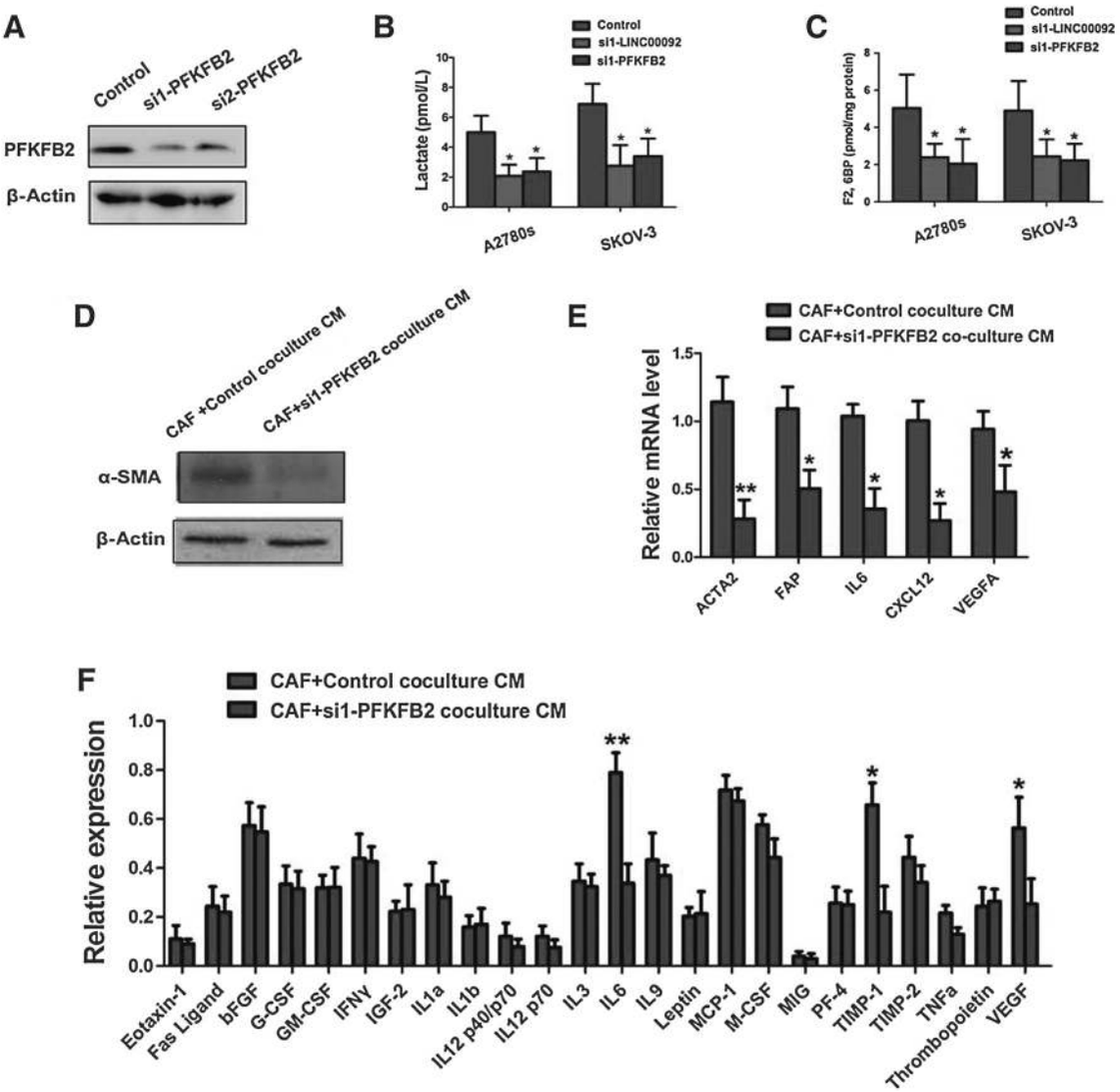

Figure 7.

Glycolytic phenotype of ovarian cancer sustains the hallmark of CAFs. **A,** Knockdown efficiencies of siRNAs targeting PFKFB2 using Western blotting analysis. **B,** Lactate production in control, LINC00092-silenced, and PFKFB2-silenced A2780s cells and SKOV-3 cells. **C,** F2,6BP production in control, LINC00092-silenced, and PFKFB2-silenced A2780s cells and SKOV-3 cells. **D,** Protein level of α-SMA in CAFs cocultured with control A2780s cells and in CAFs cocultured with PFKFB2-silenced A2780s cells. **E,** Relative mRNA levels of ACTA2, FAP, IL6, CXCL12, and VEGFA in CAFs cocultured with control A2780s cells and in CAFs cocultured with PFKFB2-silenced A2780s cells. **F,** CAFs were cocultured with control A2780s cells or cocultured with PFKFB2-silenced A2780s cells for 48 hours. The levels of various factors in culture media were measured by antibody array and were normalized to positive control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

critical for peritoneal metastasis in ovarian cancer nude mice model. These data suggest that LINC00092 functions as a downstream effector in CXCL14-high CAF-promoted ovarian cancer progression.

Enhanced glycolysis under aerobic conditions (the Warburg effect) has been a hallmark of cancer (16) and the enzymes and products involved in this process have been proved to promote cancer aggressiveness (33, 34). More interestingly, lncRNAs have been proven to be involved in glycolysis regulation in cancer. For instance, Ma and colleagues demonstrated that lncRNA GCASPC could modulate glycolysis by directly binding with pyruvate carboxylase in gallbladder cancer (17). Another report showed that lncRNA ceruloplasmin (NRCP) could serve as an intermediate binding partner between STAT1 and RNA polymerase II, leading to increased expression of downstream target genes such as glucose-6-phosphate isomerase modulating cancer glycolysis (18). In our study, we asked whether LINC00092 could also modulate glycolysis to promote ovarian cancer metastasis. Through RT-PCR and RNA pull-down assays, we identified PFKFB2 as a key glycolytic enzyme that directly interacted with LINC00092. PFKFB2 was significantly overexpressed in ovarian cancer with metastasis compared with normal ovary and ovarian cancer without metastasis, which was confirmed to be functionally important for ovarian cancer metastasis. More interestingly,

PFKFB2-induced glycolytic phenotype of ovarian cancer cells was critical for the maintenance of CAF-like features of fibroblasts, indicating a reciprocal feedback loop between CXCL14-positive CAFs and ovarian cancer cells.

In conclusion, our study demonstrated that CXCL14-positive CAFs were important in ovarian cancer metastasis, partially due to paracrine induction of LINC00092 in ovarian cancer cells. LINC00092 interacted with PFKFB2 to induce a glycolytic phenotype in ovarian cancer. These CAF-related Warburg effects in ovarian cancer, in return, were critical for the maintenance of CAF-like features, thus forming a regulatory feedback loop within tumor microenvironment. Our results provided a novel insight into the biology of ovarian cancer metastasis and raised the possibility of new options for clinical interventions for ovarian cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Zhao, G. Ji, C. Wang, L. Xu, H. Yang, Y. Xuan, Y. Yang, L. Lei, Q. Yang, W.B. Lau, B. Lau, S. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Zhao, G. Ji, X. Le, C. Wang, L. Xu, L. Lei, Y. Chen, X. Deng, S. Zhou
Writing, review, and/or revision of the manuscript: L. Zhao, W.B. Lau, B. Lau, S. Zhou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Le, L. Xu, M. Feng, Y. Zhang, W.B. Lau, T. Yi, S. Zhou
Study supervision: S. Yao, X. Zhao, Y. Wei, S. Zhou

Grant Support

This work was supported by grants from the National Natural Science Foundation of China (grant #81402396), Sichuan Science-Technology Soft Sciences Project (grant #2016ZR0086), Yi Yao Foundation (grant #14H0563) and Direct Scientific Research Grants from West China Second Hospital, Sichuan University (grant #KS021).

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Received June 10, 2016; revised October 27, 2016; accepted December 7, 2016; published OnlineFirst January 13, 2017.

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